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High-Frequency Leader Sequence Switching during Coronavirus Defective Interfering RNA Replication

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A system was developed that exploited defective interfering (DI) RNAs of coronavirus to study the role of free leader RNA in RNA replication. A cDNA copy of mouse hepatitis virus DI RNA was placed downstream of the T7 RNA polymerase promoter to generate DI RNAs capable of extremely efficient replication in the presence of a helper virus. We demonstrated that, in the DI RNA-transfected cells, the leader sequence of these DI RNAs was switched to that of the helper virus during one round of replication. This high-frequency leader sequence exchange was not observed if a nine-nucleotide stretch of sequence (UUUAUAAAC) at the junction between the leader and the remaining DI sequence was deleted. This observation suggests that a free leader RNA generated from the genomic RNA of mouse hepatitis virus may participate in the replication of DI RNA.

Several viral and cellular genes have been shown to utilize a discontinuous RNA transcription mechanism to generate mRNAs which are derived from discontinuous parts of the genomic sequences. Some of these genes, e.g., *Trypanosoma brucei* VSG gene (28, 35), *C. elegans* actin gene (8), and tobacco chloroplast ribosomal RNA genes (7), utilize a *trans*-splicing mechanism. Some others, e.g., influenza virus RNAs (31) and bunyavirus RNA (29), utilize a primer-dependent transcription to fuse part of cellular mRNAs to virus-specific mRNAs. In contrast, mouse hepatitis virus (MHV), a coronavirus, probably utilizes a virus-specific leader RNA to serve as a primer for the synthesis of subgenomic mRNAs (9). None of these discontinuous RNA transcription mechanisms, however, have been shown to operate in the synthesis of RNA with uninterrupted sequences. In the case of MHV, unlike influenza virus, there is no significant difference between subgenomic mRNAs and genomic RNA in their sequence organization, except for truncation of various internal genomic sequences. Since the leader RNA of MHV has been detected as free RNA species in virus-infected cells (1, 3), it is not unreasonable to propose that the replication of genomic RNA, which has an uninterrupted sequence, may also involve a discontinuous transcription utilizing the free leader RNA as a primer. To examine such a possibility, we developed an infectious defective interfering (DI) RNA system by using MHV DI cDNA constructs placed downstream of the T7 RNA polymerase promoter. The in vitro-transcribed RNA showed an extremely high efficiency of replication in the presence of a helper MHV. Surprisingly, most of the DI RNAs from the transfected cells have replaced the original leader sequence with that from the helper virus, thus suggesting that a leader RNA derived from the helper virus is involved in the replication of DI RNA.

MHV contains a single-stranded infectious RNA genome of more than 32 kilobases (kb) (15; C.-K. Shieh and M. M. C. Lai, unpublished data). It synthesizes in infected cells six major species of subgenomic mRNAs, which have a 3′ coterminal, nested-set structure (11, 17) and contain an identical 5′-end leader sequence of 72 to 77 nucleotides (10, 13, 34). A considerable body of evidence suggests that MHV utilizes a leader-primed transcription mechanism, in which a leader RNA transcribed from the 3′ end of the genomic-sized negative-strand template RNA (14) is used as a primer for subgenomic mRNA transcription at downstream intergenic regions (2, 32). As a result, the leader RNA can be freely exchanged between mRNAs of different coinfecting viruses (24). Furthermore, the leader sequence evolves very rapidly during virus passages in tissue culture; specifically, the number of pentanucleotide (UCUAA) repeat sequences at the 3′ end of the leader decreases upon serial passages (20). The change of leader sequences also results in the change of subgenomic mRNA species synthesized (20).

The JHM strain of MHV (MHV-JHM) has been shown to generate DI virus particles during high-multiplicity passages (19, 25). These DI viruses produce several different DI-specific RNAs (19) which can be classified into two types. One is DI RNA of nearly genomic size, which is efficiently packaged into virus particles and replicates itself even in the absence of helper virus infection (21). The other type consists of the smaller DI RNAs of the classical type. These DI RNAs require a helper virus and are inefficiently packaged into virus particles (21). One of the small DI RNAs has been studied in detail. This RNA, DIssE, is composed of three noncontiguous genomic regions, including the leader sequence and the 3′ end of the genome (22). However, it has a deletion of nine nucleotides (UUUAUAAAC) from the junction between the leader and the remaining genomic sequence. Since DI RNAs replicate more efficiently than does the standard MHV RNA (21), DI RNAs provide an opportunity for the study of MHV RNA replication. The data presented here indicate the utility of such a system. We have shown that MHV DI RNA replication involves a high-frequency, site-specific leader sequence switching. This result suggests that coronaviruses utilize a novel mechanism of discontinuous RNA replication.

MATERIALS AND METHODS

Viruses and cells. The plaque-cloned A59 strain of MHV (MHV-A59) was used as a helper virus. Serially passaged MHV-JHM stock at passage level 17 was used as the source of DI particles (19, 21). Mouse L2 cells (11) and DBT cells (4) were used for RNA transfection and propagation of viruses, respectively (21, 25).

DNA construction. (i) Construction of plasmid DE 25. The

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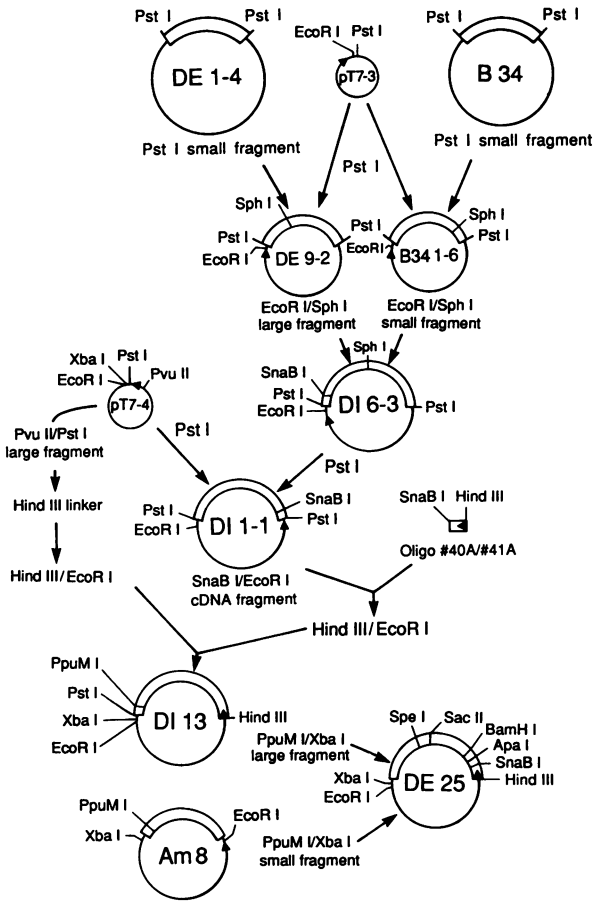


FIG. 1. Construction of the DE 25 clone of MHV DI cDNA. The details of each step are described in Materials and Methods. Closed triangles indicate the T7 promoter. The open boxes represent DI cDNA sequences.

procedures for construction are diagrammed in Fig. 1. The 1.85-kb *Pst*I fragment of clone DE 1-4, representing the 5′ end of DIssE RNA (22), was inserted into the *Pst*I site of the transcription vector pT7-3 (36). This construct was designated DE 9-2. The 1.5-kb *Pst*I fragment of plasmid B34, representing the 3′ end of DIssE, was also subcloned into pT7-3, yielding B34 1-6. The large *Eco*RI-*Sph*I fragment of DE 9-2 and the 1.1-kb *Eco*RI-*Sph*I fragment of B34 1-6 were ligated to generate DI 6-3, which contained almost the entire DIssE sequence except for a few 5′-end nucleotides but contains GC tails at both 3′ and 5′ ends of DI cDNA insert. The 2.2-kb *Pst*I fragment from DI 6-3 was subcloned into pT7-4 vector, yielding DI 1-1.

To remove the 5′-end GC tail and complete the 5′-end DI cDNA sequence, the 2.2-kb *Sna*BI-*Eco*RI fragment of the DI cDNA of DI 1-1 was ligated with a double-stranded synthetic oligonucleotide (oligo 40A/41A)

5′-AAGCTTAATACGACTCACTATAGTATAAGAGTGAATGGCGTCCGTAC-3′
3′-TTCGAATTATGCTGAGTGATATCATATTCTCACTTACCGCAGGCATG-5′

that contains the T7 promoter sequence, the 5′-end sequence of DIssE and a *Hin*dIII site at the end. After ligation, DNA was cleaved with *Hin*dIII and *Eco*RI and ligated to the 2.2-kb *Pvu*II-*Pst*I fragment of pT7-4, to which had been added a *Hin*dIII linker, and treated with *Eco*RI and *Hin*dIII. The resulting plasmid, DI 13, contained a *Hin*dIII site, which is followed by T7 promoter sequence and complete DIssE sequence such that the RNA transcript contained an additional G residue at the 5′ end (Fig. 2).

To remove the 3′-end GC tail in the DI cDNA clone, a 4.4-kb *Ppu*MI-*Xba*I fragment of DI 13 was ligated with the 0.15-kb *Ppu*MI-*Xba*I fragment of plasmid Am 8, which contains a 1.8-kb cDNA fragment of 3′ end of MHV-JHM genomic RNA in a pTZ18U vector with no GC tail (Shieh and Lai, unpublished data). The resulting plasmid, DE 25, does not contain any GC tail at either the 5′ or 3′ end of the complete DI cDNA and contains 60 nucleotides of poly(A) tail.

(ii) Construction of other DI cDNAs. To construct DI clones with different leader sequences, the 2.2-kb *Sna*BI-*Eco*RI DI cDNA fragment of DE 25 was ligated with a double-stranded synthetic oligonucleotide (oligo 52/53)

5′-AAGCTTAATACGACTCACTATAGTATAAGAGTGATTGGCGTCCGTAC-3′
3′-TTCGAATTATGCTGAGTGATATCATATTCTCACTAACCGCAGGCATG-5′,

which contains sequence identical to oligo 40A/41A, except for the underlined base pair. After ligation, DNA was cleaved with *Xba*I and the large fragment was phosphorylated and ligated to the 2.2-kb *Pvu*II-*Xba*I fragment of the pT7-4 vector, yielding plasmid DE 5. The 4.1-kb *Apa*I-*Sna*BI fragment of DE 5 was ligated with 0.22-kb *Sna*BI-*Apa*I fragments of clones F1 and F82 (32), yielding DE5-w3 and DE5-w4, respectively. The plasmids F1 and F82 are cDNA clones of the 5′ end of MHV-JHM genomic RNA and contain three and four repeats of TCTAA, respectively.

The 2.2-kb *Hin*dIII-*Eco*RI DI cDNA fragment of DE 25 was subcloned into *Eco*RI-*Hin*dIII sites of pBR322. The resulting plasmid was cleaved with *Sna*BI and ligated with the 0.4-kb *Sna*BI-*Bam*HI fragment of plasmid K 61, which contains the 0.6-kb cDNA fragment of the 5′ end of MHV-JHM genomic RNA in pBR322 and contains only one TCTAA repeat (S. Makino, unpublished data). After ligation, DNA was digested with *Apa*I and the large fragment was self-ligated, yielding plasmid DE5-w1.

The 3-kb *Sna*BI-*Spe*I fragment of DE 25 was ligated with 1.5-kb *Sna*BI-*Spe*I fragments of DE5-w3, DE5-w4, and DE5-w1, yielding DE107-w3, DE107-w4, and DE107-w1, respectively.

The 1.2-kb *Sac*II-*Dra*I DI cDNA fragment of DE 1-4 was ligated with a double-stranded synthetic oligonucleotide (oligo 78/79)

5′-AGCTTTACGTACCCTCTCTACTCTAAAACTCTTGTAGTTT-3′
3′-AATGCATGGGAGAGATGAGATTTTGAGAACATCAAA-5′

and then ligated with the 3.5-kb *Sac*II-*Hin*dIII fragment of DE 25. The 1.5-kb *Sna*BI-*Spe*I fragment of this plasmid was ligated with the 3-kb *Sna*BI-*Spe*I fragments of DE 25 and DE5-w4, yielding DE-1A and DE-2c, respectively.

The 5′-end sequences of all DI cDNA clones were confirmed by double-stranded dideoxy DNA sequencing.

RNA transcription and transfection. Plasmid DNAs were linearized by *Xba*I digestion and transcribed with T7 RNA polymerase as previously described (33). RNA transfection was done as previously described (21). Briefly, L2 cells were first infected with MHV-A59. After 1 h of virus adsorption, virus inoculum was removed and RNA was added into the culture in the presence of transfection buffer (600 μg of DEAE-dextran per ml, 0.14 M LiCl, HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.5, and 1 mM $MgCl_2$) at 0.5 μg of RNA per 0.2 ml. After 30 min of incubation at room temperature, the inoculum was removed

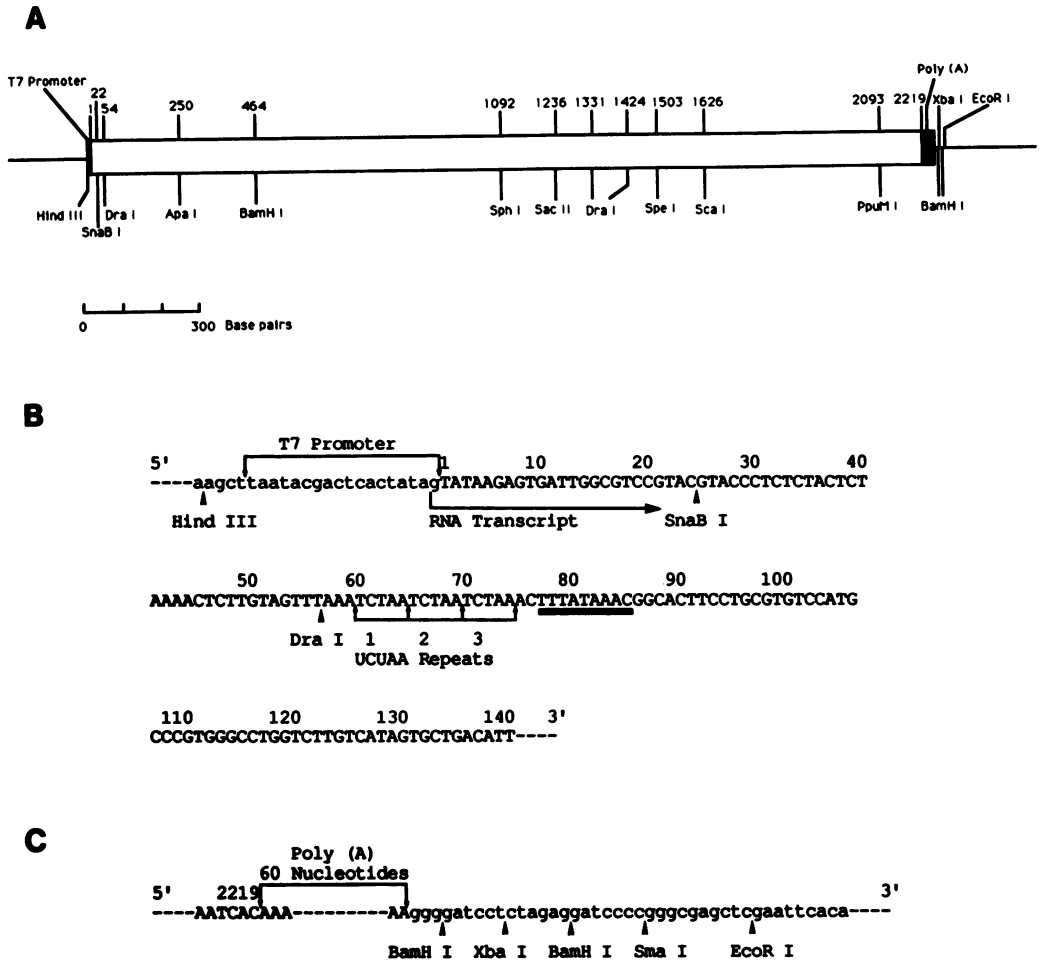


FIG. 2. Diagram of the structure of DE5-w3. (A) □, DI cDNA sequence; ——, vector sequences. Restriction enzyme sites used for plasmid DNA construction are indicated. (B) Sequence of the 5′ end region of DI cDNA of DE5-w3. The nine-nucleotide sequence (described in text) is underlined. The DI sequences are in capital letters, while the vector sequences are in lowercase letters. (C) Sequence of the 3′ end region of DI cDNA of DE5-w3 and plasmid polylinker sites.

and cultures were further incubated at 37°C in the presence of minimal essential medium containing 2% fetal calf serum.

Preparation of virus-specific intracellular RNA and agarose gel electrophoresis. Virus-specific RNAs in RNA-transfected or virus-infected cells were labeled with $^{32}P_i$ as previously described (26). The poly(A)-containing RNA was obtained by oligo(dT)-cellulose column chromatography. Virus-specific RNA was separated by electrophoresis on 1% agarose gels after denaturation with 1 M glyoxal (27). Preparative gel electrophoresis in 1% urea-agarose gels was performed as previously described (25). The RNA was eluted from gel slices by the method of Langridge et al. (16). For RNA sequencing studies, DI RNA was purified on 1% low-melting agarose gels (SeaPlaque agarose; FMC BioProducts) with the same buffer system used in the urea-agarose gel electrophoresis (25). For the elution of RNA from the gel, gel slices were first melted at 70°C and mixed with 2 volumes of TE buffer (10 mM Tris hydrochloride, pH 7.5, and 1 mM EDTA). RNAs were then extracted first with prewarmed phenol, then with phenol-chloroform and chloroform. RNA was precipitated with ethanol in the presence of 0.1 M NaCl.

Primer extension. The oligonucleotides were 5′ end labeled with [γ-^{32}P]ATP with polynucleotide kinase (30). Primer extension reactions were performed as described (22, 23). Reaction products were analyzed on 6% polyacrylamide gel containing 7 M urea.

T_1 oligonucleotide fingerprinting. ^{32}P-labeled RNA purified from infected cells or synthesized in vitro in the presence of [α-^{32}P]GTP were digested with RNase T_1; the resistant oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis as previously described (25).

RNA sequencing. The dideoxynucleotide chain termination method adapted for RNA sequencing was used (37).

RESULTS

Construction of DI RNAs capable of efficient replication in the presence of helper virus. To utilize DI RNAs for understanding the mechanism of MHV RNA synthesis and to assess the role of the 9 nucleotides (UUUAUAAAC) at the junction between leader and the remaining genome sequence, we constructed a complete DI cDNA clone (2.2 kb) which contains the complete leader sequence of MHV-JHM plus the 9 nucleotides. This DI sequence was placed downstream to the promoter for T7 RNA polymerase, so that one additional G residue is added to the 5′ end of the runoff RNA transcript (DE5-w3) (Fig. 2). The 3′ end of this DI cDNA construct contains a poly(A) sequence, which is followed by

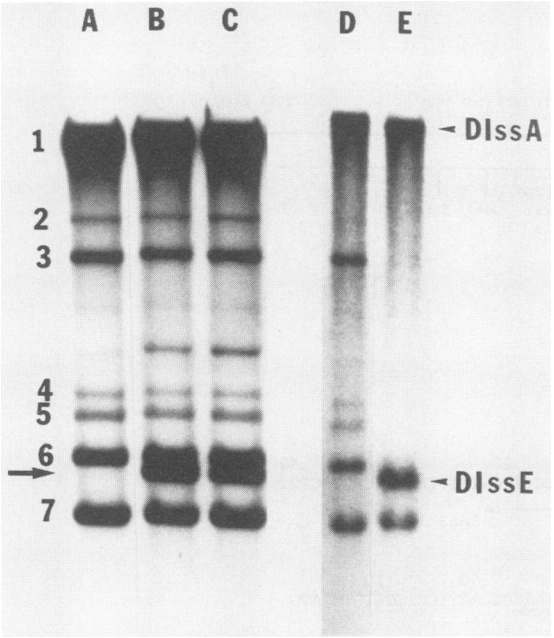


FIG. 3. Intracellular RNA synthesis after transfection with DE5-w3 RNA and infection with MHV-A59. Virus-specific RNA species were labeled with $^{32}P_i$ for 2 h in the presence of actinomycin D (2.5 μg/ml) at 5 h postinfection. RNAs were denatured with glyoxal and electrophoresed on 1% agarose gels. Lanes: A, mock-transfected and MHV-A59-infected cells; B, DE5-w3-transfected and MHV-A59-infected cells; C, passage 1 virus-infected cells; D, MHV-A59-infected cells; E, cells infected with the original DI-containing MHV-JHM virus stock. DIssA, self-replicating DI RNA species (21); DIssE, small DI RNA of the classical type (21). The arrow indicates the DI RNA. Numbers 1 to 7 represent the MHV-A59-specific mRNA species (11).

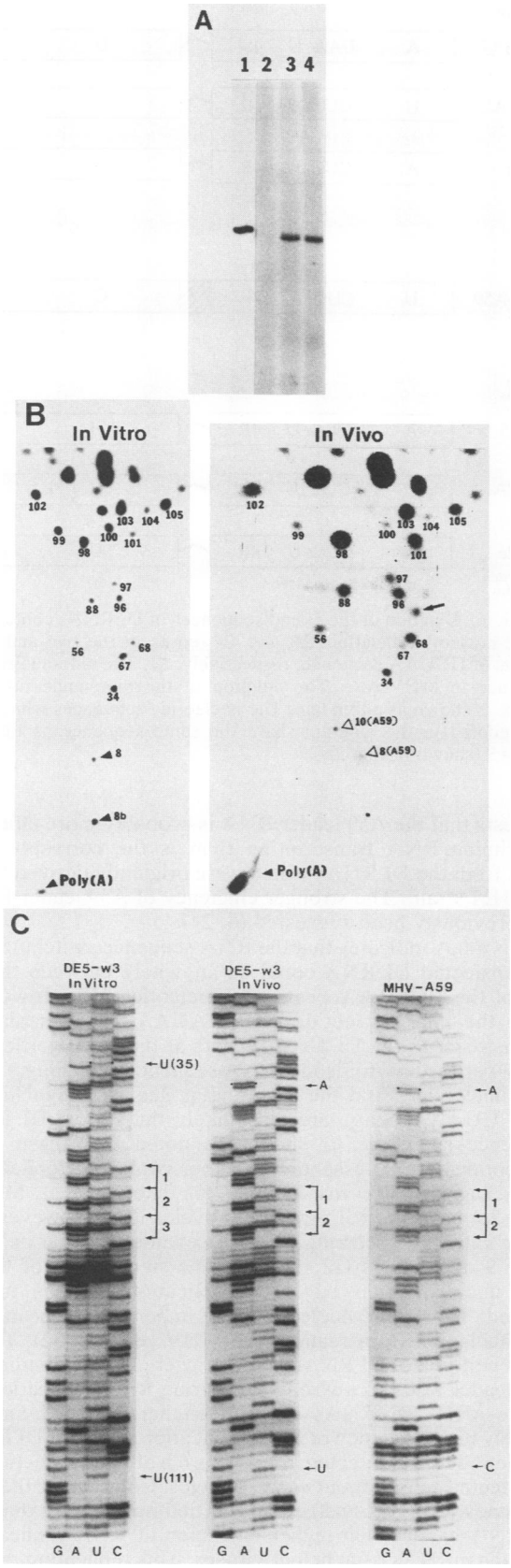
a polylinker sequence (Fig. 2). The plasmid was linearized by *Xba*I and transcribed by T7 RNA polymerase in the presence of a cap analog ($m^7G(5')ppp(5')G$). The RNA was transfected into monolayers of mouse L2 cells which had been infected with MHV-A59 at 1 h prior to transfection. MHV-A59 can be distinguished from MHV-JHM in both leader and genomic sequences (10, 32). Virus-specific RNAs were labeled with $^{32}P_i$ in the presence of actinomycin D (26) and analyzed by agarose gel electrophoresis. DI RNA of 2.2 kb was detected as early as 6 h after transfection (Fig. 3). When virus harvested from transfected cells (passage 1 virus) was used to infect L2 cells, the DI RNA was also detected in infected cells. This result indicates that this RNA could be packaged into virus particles. The amount of DI RNA synthesized increased slightly after an additional passage; however, further virus passages did not increase the amount of DI RNA appreciably (data not shown). The additional RNA which migrated between mRNA 3 and mRNA 4 is a minor mRNA frequently associated with MHV infection (26). These data thus demonstrated that in vitro-synthesized DE5-w3 RNA replicated efficiently in transfected cells in the presence of a heterologous helper virus, MHV-A59.

Change of 5′-end sequence of DI RNA in transfected cells. Since the leader sequences of mRNAs can be exchanged freely between different viruses during coinfection (24) and the leader sequence evolves rapidly during virus passages in tissue culture (20), we examined whether any alteration occurred at the 5′ end of DI RNA species in transfected cells. We first carried out primer extension studies by using an oligonucleotide (oligo 1) complementary to nucleotides 123 to 140 from the 5′ end of genomic RNA (32), representing sequences slightly downstream to the leader RNA. The 5′ end-labeled primer was hybridized to purified DI RNA and extended with reverse transcriptase (Fig. 4A). Surprisingly, the primer extension product of DI RNA from transfected cells was slightly smaller than that of the in vitro-synthesized DI RNA. In cells infected with helper virus alone, RNA obtained from the 2.2-kb region did not yield any primer extension product (Fig. 4A, lane 2). These data suggested that the 5′ end of DI RNA underwent some sequence alterations in transfected cells.

To examine whether the sequence alteration was limited to the 5′ end of DI RNA or involved the entire DI genome, we examined the DI RNA by T_1 oligonucleotide fingerprinting. The DI RNA from cells infected with the passage 1 virus and in vitro-synthesized DI RNA were digested with RNase T_1 and separated by two-dimensional polyacrylamide gel electrophoresis. The overall fingerprint patterns of both DI RNAs were similar, although the spot intensity was variable, probably because of differences between in vivo and in vitro labeling and also because of degradation of in vivo-labeled RNA, which required extensive purification. However, there were several differences. The oligonucleotides 8 and 8b in the in vitro-synthesized RNA were not present in the DI RNA from infected cells. These two spots were derived from the leader sequence of MHV-JHM (26), which is the parent of DI RNA. On the other hand, DI RNA from passage 1 virus-infected cells contained two T_1 spots, 10 and 8, which were not present in the in vitro-synthesized RNA. These two spots are identical to the leader oligonucleotides of the helper virus MHV-A59 (10, 11), indicating that the DI RNA in transfected cells has lost the original leader sequence and replaced it with the leader sequence of the helper virus MHV-A59. The rest of sequence did not undergo gross sequence alteration. There is an additional oligonucleotide which was not present in the in vitro-transcribed RNA. This spot has been occasionally detected in the naturally occurring DI RNA (19).

The origin of the 5′ end of DI RNA was further confirmed by RNA sequencing with a primer (oligo 56) complementary to nucleotides 172 to 188 from the 5′ end of the genomic RNA. The result of RNA sequencing is shown in Fig. 4C. The in vitro-transcribed DI RNA contained a U residue at position 35 and three repeats of a UCUAA sequence at the 3′ end of leader. On the contrary, the DI RNA from passage 1 virus-infected cells contained an A at position 35 and two repeats of the UCUAA sequence, which is identical to the leader sequence of MHV-A59 (10). Both DI RNAs had a U at nucleotide 111, identical to the parent MHV-JHM (32), whereas MHV-A59 had a C at this position, indicating that sequence alteration of the DI RNA in transfected cells occurred upstream of the nucleotide 110. These data thus demonstrated that the leader sequence of DI RNA was replaced by the leader sequence of the helper virus MHV-A59.

Several additional DI RNAs switched the leader sequence with the helper virus during replication. Since we have previously demonstrated that the number of the UCUAA repeats in the MHV genomic RNA decreased after serial virus passages in tissue culture (20), the change of the 5′ end sequence of DI RNA in transfected cells could be explained as a site-specific mutation at nucleotide 35 (U→A) and the concurrent reduction of the number of the UCUAA repeats in DI RNA. To rule out this possibility, we have prepared



several additional DI cDNA constructs. The 5′ end structures of the transcripts are shown in Fig. 5. Both DE5-w4 and DE5-w1 are identical to DE5-w3 except for the number of UCUAA repeats. Other constructs, DE107-w4, DE107-w3, and DE107-w1, contain an A at nucleotide 12. The DI RNAs transcribed from these constructs were transfected as described above. The 5′ end sequences of all of the DI RNAs obtained from passage 1 virus-infected cells are identical to the leader sequence of the helper virus MHV-A59 until nucleotide 111, where all the DIs retained the U of the original DI (Fig. 5). We therefore concluded that all of the DI RNAs switched the leader sequence with the helper virus.

A nine-nucleotide deletion eliminated the leader RNA switching. We have previously failed to detect leader RNA switching between a naturally occurring DI RNA and MHV-A59 (21). Since the naturally occurring DI RNA has a deletion of nine nucleotides at the junction between the leader and the remaining genomic sequence and has three nucleotide substitutions within leader sequence compared with the standard MHV-JHM (22), we speculated that these sequence differences between the naturally occurring DI RNA and the in vitro-constructed RNAs used here might have accounted for the difference in the leader RNA switching. Therefore, we prepared three different DI cDNA constructs which contained a deletion of the nine nucleotides (Fig. 6). The DI RNAs from passage 1 virus-infected cells retained the leader sequence of the input DI RNAs (Fig. 6). Fingerprinting analysis of DE 25 RNA from passage 1 virus-infected cells also showed that sequence change did not occur (data not shown). Since the only difference between DE-1A and DE5-w4 and between DE-2c and DE107-w4 RNAs is the absence or presence of the nine nucleotides, we concluded that the presence of the nine nucleotides located at the junction between the leader and the remaining DI sequence is crucial for the leader sequence exchange with the helper virus during DI RNA replication.

DISCUSSION

In the present study, we have demonstrated that the leader sequence of MHV DI RNAs switched to that of the helper

FIG. 4. Comparison of DI RNAs transcribed in vitro and obtained from transfected cells. (A) Primer extension analysis of the 5′ end of DI RNAs. A synthetic oligonucleotide, complementary to the nucleotides 123 to 140 from the 5′ end of the MHV-JHM genome was ^{32}P-labeled at the 5′ end, hybridized to purified DI RNA, and extended with reverse transcriptase. The product was electrophoresed on 6% polyacrylamide gels containing 7 M urea. Lanes: 1, in vitro-synthesized DE5-w3 RNA; 2, the region corresponding to a 2.2-kb region from mock-transfected and MHV-A59-infected cells; 3, 2.2-kb DI RNA from DE5-w3-transfected and MHV-A59-infected cells; 4, 2.2-kb DI RNA from passage 1 virus-infected cells. (B) T_1 oligonucleotide fingerprints of DI RNA. [α-^{32}P]GTP-labeled, in vitro-synthesized DE5-w3 RNA (A) and ^{32}P-labeled DI RNA from passage 1 virus-infected cells (B) were digested with RNase T_1, and oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis as previously described (25). The numbering of oligonucleotides is according to the previous report (19). Closed triangles in panel 1 and open triangles in panel 2 indicate the leader-specific T_1 spots (described in Results). The arrow in panel 2 indicates an additional oligonucleotide absent from in vitro-transcribed RNA. (C) Sequence of the 5′ end of DI RNA, in vitro and in vivo, and MHV-A59 genomic RNA. RNA sequence was obtained with oligo 56 used as a primer. Only the nucleotides diverged between DI RNAs and MHV-A59 genomic RNA are indicated. Numbers 1 to 3 indicate the number of UCUAA repeats. The in vivo DE5-w3 RNA was from passage 1 virus-infected cells.

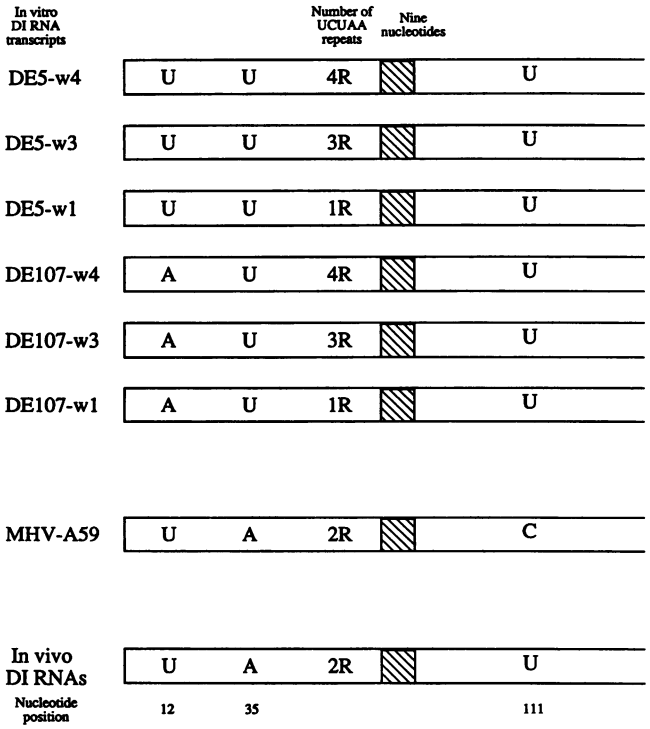


FIG. 5. Diagram of the 5′ end sequences of DI RNAs synthesized in vitro and obtained from passage 1 virus-infected cells. The nucleotide sequences which are not denoted share the same sequence as that of DE5-w3 (shown in Fig. 2B). 1R, 2R, 3R and 4R represent one, two, three and four repeats of UCUAA sequence, respectively. ▧, Nine-nucleotide sequences (UUUAUAAAC). In vivo DI RNAs represents the structure of DI RNAs obtained from passage 1 virus-infected cells irrespective of the input DI constructs.

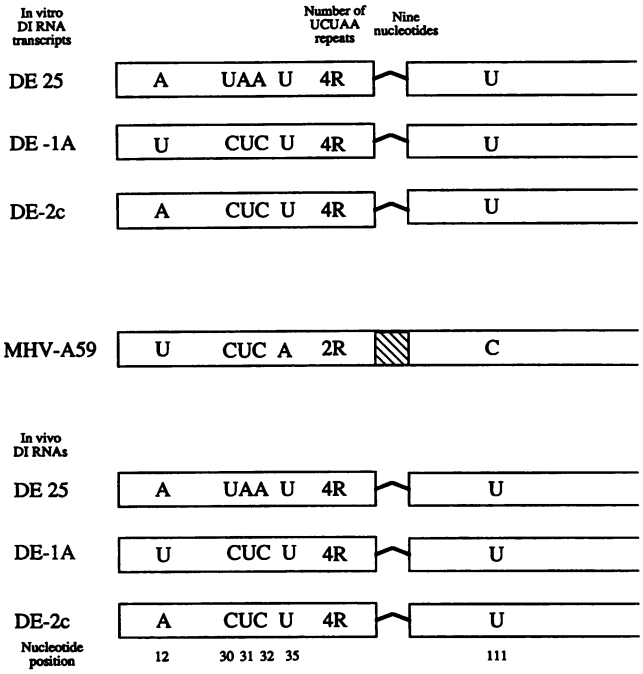


FIG. 6. Diagram of the 5′ end sequences of DI RNAs containing a nine-nucleotide deletion. 2R and 4R represent the two and four repeats of UCUAA sequence, respectively. ▧, The nine-nucleotide sequence in MHV-A59. The deletion of the nine-nucleotide sequence is shown as a thin line. The nucleotide sequences which are not denoted in the diagram share the same sequence as that of DE5-w3 (shown in Fig. 2B).

virus during DI RNA replication. This leader sequence switching requires the presence of a nine-nucleotide sequence at the junction between leader RNA and the remaining genomic sequence. Since only one round of virus replication was required for the leader RNA switching and the majority of RNA in the DI RNA-transfected cells acquired the leader RNA of the helper virus, this leader RNA switching must have occurred very early in the DI replication process and at a very high frequency. This result is most consistent with the interpretation that a free leader RNA derived from the helper virus is involved in the replication of DI RNA. We have previously shown that leader RNA species can be freely exchanged between subgenomic mRNAs of two MHVs during mixed infection (24). These two studies suggest that both transcription and replication of MHV RNAs may involve a free leader RNA species.

The presence of free leader RNA species in MHV-infected cells has previously been documented (1, 3). These free leader RNA species most likely result from termination or pausing of transcription at a stable secondary structure located at the 3′ end of the leader sequence (32). This free leader RNA from the helper virus could conceivably be used for DI RNA replication. The high frequency of this leader sequence switching and the isolation of RNA recombinants which have a crossover site at the 3′ end of leader sequence (5, 6) suggest that a free leader RNA may participate in the normal DI RNA replication process. The finding that almost all of the DI RNA species in transfected cells have acquired the leader sequences of the helper virus MHV-A59 further suggests that the A59 leader RNA is probably more efficient in priming RNA transcription than is the corresponding RNA from the DI RNAs which were originally derived from the JHM strain. The stronger efficiency of A59 leader RNA has previously been suggested (6, 24).

This study indicates that the RNA sequence switching site in transfected DI RNA could be anywhere between the 3′ end of the UCUAA repeats and nucleotide 110. However, since the nine nucleotides (UUUAUAAAC) immediately downstream to the UCUAA repeats at the 3′ end of leader RNA were crucial for leader RNA sequence switching, these nine nucleotides and the surrounding sequences, including the UCUAA repeats, are most likely the site of DI RNA sequence switching. It should be noted that these nine nucleotides are not essential for either viral RNA replication or packaging, since viruses with this deletion (e.g., MHV-JHM-2c) have normal replication cycles (23). However, all of the naturally occurring DI RNAs examined so far deleted these 9 nucleotides (12, 19, 22). The significance of these nine nucleotides in DI RNA replication remains to be studied. These nine nucleotides are imperfectly repeated at two nucleotides upstream in the MHV genome (32). Thus, the generation of DI RNA may be triggered by the binding of free leader RNA to a wrong site during RNA replication.

The MHV DI RNAs constructed here showed an extremely high efficiency of RNA replication. A major DI RNA species could be detected as early as 6 h after transfection in transfected cells without virus passages. Considering the low efficiency of RNA transfection, this finding indicates that the DI RNA has a much higher efficiency of RNA replication than the mRNAs from helper viruses. This replication rate is even higher than that of comparable DI RNA of Sindbis

virus, which requires several cycles of virus passages before DI RNAs could be detected (18). Thus, MHV DI RNA could be a potentially useful vector for expressing foreign genes in mammalian cells. It should be noted that in either DI RNA transfected or passage 1 virus-infected cells, the helper virus RNA synthesis was not inhibited. This was probably due to the low efficiency of RNA transfection and lack of specific packaging signals in these DI RNAs. We have previously shown that DIssE RNA is packaged into virion particles nonspecifically and at a very low efficiency (21). Nevertheless, whatever the small amount of RNA packaged is apparently enough to replicate into a major RNA species. The addition of a packaging signal to these DI cDNA constructs could further increase its utility as an expression vector.

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